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Lineage-related and particle size-dependent cytotoxicity of chitosan nanoparticles on mouse bone marrow-derived hematopoietic stem and progenitor cells

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ABSTRACT

Chitosan nanoparticles (CSNPs) have potential applications in stem cell research. In this study, *ex vivo* cytotoxicity of CSNPs on mouse bone marrow-derived (MBMCs) hematopoietic stem and progenitor cells (HSPCs) was determined. MBMCs were exposed to CSNPs of different particle sizes at various concentrations for up to 72 h. Cytotoxicity effect of CSNPs on MBMCs was determined using MTT, Live/Dead Viability/Cytotoxicity assays and flow cytometry analysis of surface antigens on HSCs (Sca-1$^+$), myeloid-committed progenitors (CD11b$^+$, Gr-1$^+$), and lymphoid-committed progenitors (CD45$^+$, CD3e$^+$). At 24 h incubation, MBMCs' viability was not affected by CSNPs. At 48 and 72 h, significant reduction was detected at higher CSNPs concentrations. Small CSNPs (200 nm) significantly reduced MBMCs' viability while medium-sized particle (~400 nm) selectively promoted MBMCs growth. Surface antigen assessment demonstrated lineage-dependent effect. Significant decrease in Sca-1$^+$ cells percentage was observed for medium-sized particle at the lowest CSNPs concentration. Meanwhile, reduction of CD11b$^+$ and Gr-1$^+$ cells percentage was detected at high and intermediate concentrations of medium-sized and large CSNPs. Percentage of CD45$^+$ and CD3e$^+$ cells along with ROS levels were not significantly affected by CSNPs. In conclusion, medium-sized and large CSNPs were relatively non-toxic at lower concentrations. However, further investigations are necessary for therapeutic applications.

1. Introduction

Chitosan has considerable potential in medical applications, including cellular delivery systems and tissue engineering based on its ability to interact with negatively charged molecules within cells (Richardson et al., 1999). Chitosan has muco-adhesive properties allowing sustained interaction with membrane epithelia, and thus promoting efficient uptake of drugs (Qaqish and Amiji, 1999). In addition, chitosan has the ability to open intercellular tight junctions and to facilitate transport of drugs into cells (Illum et al., 2001). The mechanism involved in the action of chitosan as a delivery system is the combined effects of muco-adhesion and tight-junction regulation (Nicolaas et al., 1997). The preparation of CSNPs by the ionic gelation method is simple and requires no use of organic solvents, which minimizes the side effects from its use (Pan et al., 2002). Despite the benefits of chitosan for delivering molecules to cells and its potential in stem cell applications, evaluation of biological adverse effects associated with exposure to CSNPs remains limited.

Nanotechnology has been widely investigated over the years, and studies concerning the toxic effects of nanomaterials and their interaction within biological systems have been reported (Hood, 2004; Yang et al., 2009; Klein, 2007; Maynard et al., 2006; Unfried et al., 2007). Despite the plethora of studies, most have

Abbreviations: CSNPs, Chitosan nanoparticles; DHE, dihydroethidium; FITC, fluorescein isothiocyanate; H_2O_2, hydrogen peroxide; HE, hydroethidine; HSPCs, hematopoietic stem and progenitor cells; MBMCs, mouse bone marrow-derived; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PDI, polydispersity index; RNAi, RNA interfering; ROS, reactive oxygen species; TPP, tripolyphosphate.

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not provided any clear evidence of the correlation existing between the type of cells and the nanoparticles characteristics. Moreover, the safety aspects of nanoparticulate systems remain a constant concern due to the possibility of unpredictable adverse effects associated with these nanoparticles (Nafeea et al., 2009). A number of cationic polymers were reported to be toxic due to electrostatic interactions with plasma membrane and/or negatively charged cellular components and proteins (Illum et al., 2001). Thus, it has been postulated that the cytotoxicity effects of nanomaterials could be influenced by their physiochemical characteristics such as particle size, size distribution, surface charge, and presence of functional groups on the surface. Nonetheless, a comprehensive understanding on the effect of particle size, shape, composition and aggregation-dependent interactions of nanostructures with biological systems, particularly in hematopoietic stem cells (HSCs), is still lacking.

HSCs are multi-potent stem cells responsible for blood cell generation with the ability to self-renew, differentiate into cells of all hematopoietic lineages and undergo apoptosis to maintain the hematopoietic system normal pool (Suda et al., 2005). HSCs differentiate into two majorly-distributed lineages, namely, myeloid lineage (monocytes, macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, and dendritic cells) and lymphoid lineage (T cells, B cells, and natural killer cells) (Akashi et al., 2000). This distinctive ability of HSCs to differentiate into these two lineages makes them suitable for hematopoietic regeneration as well for the support of tissue repair and restoration in non-hematopoietic tissues (Suda et al., 2005). HSCs have been used in various clinical applications and have shown encouraging outcomes in the treatment of human diseases, ranging from bone marrow failure syndromes to clonal neoplastic disorders (Hanson et al., 2003). Manipulation of HSCs in the revival of non-hematopoietic organs was also reported due to their ability to differentiate into a variety of non-hematopoietic cell types *ex vivo* (Akashi et al., 2000).

Nanoparticles have been applied to stem cell research in the context of cellular delivery of DNA, RNA interfering (RNAi)-based molecules, proteins, peptides, and drugs for stem cell differentiation (Moghimi et al., 2005; Muschler et al., 2004). However, available information on nanotoxicology, particularly in relation to stem cells biology, remains insufficient and deserves further investigation. To date, an extensive study demonstrating the safety and biocompatibility of CSNPs on HSCs has not been reported. Therefore, a systematic study concerning CSNPs toxicological profile is needed to evaluate the potential interference of CSNPs with the biological properties of stem cells. In this study, different sizes of CSNPs were investigated for their cytotoxic effect in MBMCs by determining cell viability, phenotypic expression of surface antigens for HSCs (Sca-1^+), myeloid-committed progenitors ($CD11b^+$, Gr-1^+), and lymphoid-committed progenitors ($CD45^+$, $CD3e^+$), as well as the production of reactive oxygen species (ROS).

2. Methods

2.1. Ethics statement and animal care

All mice were housed and handled in strict accordance with the recommendations from Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC). All animal experiments were carried out with the UKMAEC approval number FF/2012/HALIZA/21-NOV./476-NOV.-2012-JAN.-2014-AR-CAT2. Standard practices of animal care and use were applied to all the animals used in this project. Research protocols were approved by UKMAEC.

2.2. Preparation and characterization of CSNPs

CSNPs of different particle sizes were prepared using the ionotropic gelation method (Katas and Alpar, 2006). This method involves mixing of 3.0 mL chitosan solution of various concentrations (0.2, 0.3, 0.4, 0.5, and 0.6 w/v %; chitosan was dissolved in 2 M acetic acid) and 1.2 mL TPP solution (0.1% w/v in distilled water) under a continuous magnetic stirring at 700 rpm for 1 h at room temperature. FITC-labeled CSNPs (FITC-CSNPs) for cellular uptake study were prepared according to a previously reported procedure (Ma and Lim, 2003). CSNPs were collected by ultracentrifugation (Optima L-100 XP, Beckman–Coulter, California, USA) at 35,000 rpm, 10 °C for 30 min and the pellets were dispersed in deionized distilled water. Physical characteristics (particle size and its distribution, polydispersity index (PDI), and zeta potential) of CSNPs were measured and characterized using a Malvern Zetasizer (Nano ZS®, Malvern Instruments, UK). All formulations were characterized immediately after preparation. Labeling efficiency (% w/w FITC to FITC-chitosan) was determined using a fluorometric technique by measuring the fluorescence intensity of FITC-chitosan solution against standard solutions containing 0–80 ng/mL of FITC in PBS (Ma and Lim, 2003; Huang et al., 2005). The labeling efficiency was calculated as percentage FITC (mg) over the total weight of FITC-chitosan (mg) in the sample.

2.3. Cell culture

MBMCs were isolated from ICR strain mice origin using the standard "flushing" technique (Dobson et al., 1999). Mice were sacrificed by the cervical dislocation method as approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC) with the approval number FF/2012/HALIZA/21-NOV./476-NOV.-2012-JAN.-2014-AR-CAT2. Bones (femurs and tibiae) were flushed with 2% DMEM and single-cell suspensions of the cells were made using a 40 μm cell strainer. MBMCs were then centrifuged at 2500 rpm for 7 min and the collected cells (pellets) were re-suspended in 5 mL of growth medium DMEM supplemented with 10% FBS, 2% Pen-Strep (100 U/mL Penicillin, 100 μg/mL Streptomycin), 100 ng/mL SCF, 5 ng/mL interleukin 3 (IL-3) and 10 ng/mL interleukin 6 (IL-6). The isolated MBMCs were then cultured in 75 cm^2 cell culture flasks and grown to a density of 1×10^6 cells/mL of growth medium at 37 °C in 5% CO_2 incubator for 24 h.

2.4. MTT assay

The effects of CSNPs on MBMCs viability were assessed by MTT assay following a previously described protocol (Mosmann, 1983). Briefly, MBMCs were seeded at 1×10^4 cells/well in a 96-well plate and cultured in the presence of CSNPs with concentrations ranging from 31.25 to 1000 μg/mL for 24, 48, and 72 h. The cells were incubated at 37 °C in a 5% CO_2 incubator. At the pre-determined exposure times, 100 μL of MTT solution (1 mg/mL) was added to the culture and the cells were further incubated for 4 h at 37 °C. Following aspiration of the MTT solution, the formazan crystals produced were solubilized with 150 μL of DMSO for 10 min at 37 °C and quantified by measuring absorbance at 570 nm using a microplate reader (BioRad, USA) and the defined optical density (OD) correlates with the viability of the cells in culture.

2.5. Live/dead assay

The MBMCs viability was further assessed using the Calcein AM/EthD-1 Live/Dead Viability/Cytotoxicity assay (Molecular Probes). Calcein AM specifically stains live cells due to enzymatic conversion

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